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1) J Chromatogr 1990 Feb 23;525(2):297-306
Purification of urokinase by combined cation exchanger and affinity chromatographic cartridges.
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PURIFICATION OF UROKINASE BY COMBINED CATION EXCHANGER AND AFFINITY CHROMATOGRAPHIC CARTRIDGES

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SUMMARY

Crude urokinase from human urine processed through foam flotation and ammonium sulfate precipitation containing 720 National Health Institute Committee on Thrombolytic Agents U/mg activity was purified by an SP cation exchanger followed by a zinc-chelated affinity chromatographic cartridge. The cartridges were of a radial-flow type formed by using acrylic and cellulose composite matrices. The high rigidity of the matrix structure permits fast flow of protein solutions (liters per minute) and thus allows processing of a large volume of crude urokinase under low operating pressures. A greater than six-fold increase in specific enzyme activity of urokinase was achieved by adsorbing and eluting 1 l of a 3 mg/ml crude urokinase solution on an SP cartridge. The eluent was further purified by passing through a zinc-chelated affinity cartridge to achieve greater than a eighteen-fold increase in urokinase specific activity. This report demonstrates the combined use of a cation exchanger with zinc-chelated chromatographic cartridges in purifying urokinase on a relatively large scale. The relationship between the amount of zinc chelated in the matrix to its effect on urokinase purification is also discussed.

INTRODUCTION

Urokinase (UK) (EC 3.4.99.26) is a plasminogen-activating enzyme present in urine [1–4] and produced in the kidney [5,6]. Two immunologically and biochemically distinct types of human plasminogen activator are currently known: tissue-type plasminogen activator (t-PA) [7,8] and UK-type plasminogen activators. UK, first identified in human urine, is commercially isolated from urine for therapeutic use as a thrombolytic agent. Extensive work has been done on its purification [9,10], but commercially available crude UK is still contaminated with degradation fragments and other unrelated proteins.

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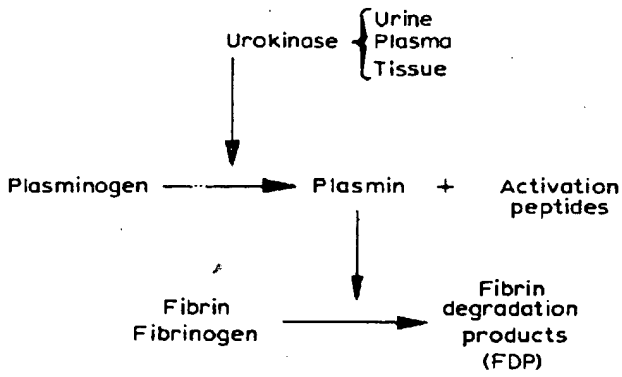

Fig. 1. Schematic representation of urokinase in fibrinolytic system for blood clot dissolution.

The UK-related proteins in human urine consist of ca. 25% one-chain UK (10–20 μg/l) and 75% two-chain UK (40–50 μg/l), the bulk of which is complexed to an inhibitor [1]. Even in freshly voided urine, most of the UK-related antigen is already converted into two-chain UK with a relative molecular mass of 45 000–50 000 and is composed of two polypeptide chains joined by a single disulfide bridge [11]. The UK activates plasminogen to plasmin by a cleavage of the Arg^{560}–Val^{561} peptide bond [12]. The plasmin formed is responsible for the lysis of fibrin via the hypothetical mechanism shown in Fig. 1. On isoelectric focusing, the high-molecular-mass two-chain UK showed a single peak at pH 9.7 and the low-molecular-mass UK, of multiple isoelectric subforms, ranged between 6.85 and 9.37 [13]. This suggests that UK can be adsorbed by a cationic matrix, such as the sulfopropyl (SP) group, and eluted by a pH and salt gradient for its purification.

Purification methods described so far include a series of conventional chromatographic procedures [14,15] and/or the use of affinity columns carrying analogues of UK substrates, such as benzamidine [16,17] and agmatine [18]. Other affinity ligands, such as monoclonal antibodies [19] and metal-chelating (of the imidodiacidic acid type) have also been reported [20,21]. Most of this published work was performed in a column packed with a soft gel operated at a low flow-rate and was suitable for purifying only milligrams or less of UK. In order to process a large volume of protein solution in an industrial operation, we have developed a dimensionally stable composite matrix of cellulose grafted to an acrylic polymer to withstand high liquid flux. The matrix was then installed in a cartridge form to facilitate operation and scale-up [22,23]. The purification of crude UK described in this paper demonstrates that such fast-flow cartridges are effective for purifying biological products in a large scale.

EXPERIMENTAL

Materials

Lyophilized crude UK derived from foamed urine received from Taitong Enterprise (Taipei, Taiwan) was used as the starting material. The enzymic ac-

tivity was determined as 720 National Health Institute Committee on Thrombolytic Agents (CTA) U/mg by the following assay methods.

Assay methods

Two general types of assay were applied, based on (1) the specific ability of UK to activate plasminogen by caseinolytic method and (2) the amidolytic activity of UK toward a synthetic substrate.

Specific ability of UK as plasminogen activator. The principle of this method is to incubate UK with an excess of plasminogen, so that the amount of plasmin formed is proportional to the UK concentration. Casein was used as the substrate and Tris–HCl as the buffer for the plasmin assay. One CTA plasmin unit releases 0.1 μequiv. of tyrosine per min from Lot GC 1-16 α-casein, which the Subcommittee of the National Heart Institute Committee of Thrombolytic Agents used as the standard substrate. All the standard samples of α-casein, plasminogen, plasmin and UK were kindly provided by Dr. A.J. Johnson of the New York Medical Center. We followed his procedure for the assay of UK by measuring the release of tyrosine at O.D. 275 nm using a standard calibration curve [24]. One CTA UK unit activates ca. 0.19 CTA units of plasminogen.

Amidolytic assay. This assay uses a synthetic substrate directly: no other protein, such as plasminogen, fibrinogen or casein, is used. The substrate used is Kabi 2444 (L-pyroglutamyl-glycyl-arginine-*p*-nitroanilide hydrochloride). The method for the determination of activity is based on the difference in optical density between the *p*-nitroanilide (pNA) released from the substrate and the original. The rate of pNA formation per second at 405 nm is proportional to the enzymic activity and is determined with a spectrophotometer. An activity of *A* per min of 0.05 at 37°C is obtained by using 0.3 m*M* substrate in Tris buffer at pH 8.8, corresponding to 40 CTA U/ml of UK standard received from Dr. Johnson or 25 ploug U/ml of UK standard received from The National Institute for Biological Standards and Control (London, U.K.). A standard calibration curve made from a series of dilutions is sensitive to less than 5 CTA U/ml.

Protein concentration determination

The amount of protein was determined by the method of Lowry et al. [25], with bovine serum albumin as a standard. During the purification procedure, the protein concentration was monitored by absorbance at 280 nm using 16.1 as the molar absorptivity for high-molecular-mass UK.

Chromatographic matrices and cartridge device

The chromatographic solid matrices were a composite of cellulose with grafted acrylic polymers, produced by developing a glycidyl methacrylate polymer in the presence of dispersed cellulosic fiber, followed by an in situ covalent binding of the acrylic polymer to the cellulose [22]. The bi-component fiber thus

formed consisted of a cellulosic core as the mechanical support and the acrylic sheath as a chemical functional group carrier. The composite fiber was then derivatized to the required functional groups, such as sulfopropyl (SP), through a sodium sulfite and glycidyl reaction [26], or dicarboxyl groups as metal chelators. The composite fiber carrying specific functional groups was then fabricated in paper form. To form a radial-flow cartridge, a thin paper sheet was spirally wound on a central slotted plastic core. A polypropylene outer sleeve was added to the multi-layered module for protection, and end-caps were heat-sealed to the top and bottom of the cartridge. The housing of the cartridge provides routes that radially distribute the influent solution to the peripheral surface of the cartridge. After passing through the solid matrix, the solution flows along the central duct and leaves the housing [23,27,28]. All the chemicals were purchased from Aldrich in reagent grade and the monomers were received from Sartomer (West Chester, PA, U.S.A.)

Zinc chelate preparation

Zinc chelate matrix was prepared by coupling iminodiacidic acid to the glycidyl groups grafted on cellulose according to the following mechanisms (Fig. 2) and the work of Porath [20]. The fibrous matrix was dispersed in 0.2 *M* sodium hydroxide solution, in 10% consistency, agitated for 1 h and then vacuum-filtered and washed with deionized water until neutrality. The washed fiber was re-dispersed in 10 m*M* zinc nitrate solution (pH 6.0) under agitation for 2 h. The filtering and washing cycle was repeated twice to ensure that all unbound metal ions were washed away from the matrix. A Perkin-Elmer atomic absorption spectrophotometer was used to measure the metal ions.

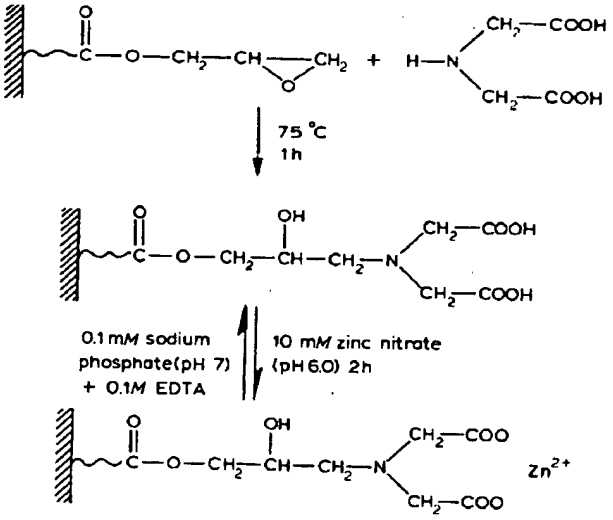

Fig. 2. Synthesis of zinc-chelated composite matrix containing eight atom spacer arms.

Study on the amount of zinc chelated to the matrix to the binding of UK

Zinc solutions, 100-ml samples each of a different molarity, were prepared by dissolving zinc nitrate hexahydrate in deionized water (pH 5.0) and mixing with 0.25 g of the matrix with overnight shaking at room temperature. The zinc-chelated matrices were then centrifuged and washed until no amount of zinc detectable by atomic absorption measurement was left in the washing solution. The concentration difference between the original and final zinc solutions was expressed as the amount chelated to the matrix; 50 mg of the oven-dried matrix was redispersed in 10 ml of a crude UK solution prepared by dissolving 4 mg/ml UK in 0.05 *M* borate with 2 *M* sodium chloride plus 0.01% Tween 80 (pH 8.2), and tumbling for 30 min at room temperature to ensure sufficient mixing. The matrix was pelleted under centrifugation, and the loss of UK activity due to adsorption by the zinc-chelated media was recorded.

SP cartridge procedure

A 500-ml aliquot of 0.1 *M* tribasic sodium phosphate was applied to activate the cartridge, followed immediately by 100 ml of 0.1 *M* acetic acid at a flow-rate of 100 ml/min to bring it back to neutral. The cartridge was then equilibrated using 0.05 *M* sodium acetate with 0.01% Tween 80 (pH 4.5). A 1-l volume of a crude UK sample, in equilibration buffer, was applied, and all unbound material was washed to a baseline with the equilibration buffer. A pre-elution step was run using equilibration buffer with 0.4 *M* sodium chloride. The elution was completed using the equilibration buffer with 1.0 *M* sodium chloride. During the purification procedure 100-ml fractions were collected. Protein concentrations were followed using absorbance measurements at 280 nm, and UK activities were measured using the amidolytic assay and checked with the caseinolytic assay from time to time.

Zinc-chelated cartridge procedure

The zinc-chelated cartridge was equilibrated with 0.05 *M* borate, 0.2 *M* sodium chloride and 0.01% Tween 80 (pH 8.2) buffer. A 180-ml aliquot of combined fractions 30 and 31, from the SP purification step, was dialyzed against equilibration buffer with 10% glycerol and applied to the equilibrated zinc-chelated cartridge at a flow-rate of 50 ml/min. All unbound material was washed to the baseline using equilibration buffer. An elution step using equilibration buffer containing 0.05 *M* imidazole followed. During the purification procedure, fractions of 25 ml were collected. The same procedures and methods as above for monitoring protein concentration and UK activity were followed.

RESULTS AND DISCUSSION

The use of UK from urine as a thrombolytic agent has a long history [9] and is still in popular practice [1,29]. Efforts continue to improve large-scale pro-

duction of UK for high yield of enzymic activity. Our interest in providing a fast flow and economical process was kindled by the request of a Taiwan pharmaceuticals producer, who wished to have more than a ten-fold increase in UK specific activity for his product. The crude UK we received was directly processed from human urine through foam flotation and ammonium sulfate precipitation. It was in a yellowish powder from, containing 720 CTA U of enzyme activity per mg protein measured by the amidolytic assay. Since the sample contained other proteins and also salts as contaminants, the specific activity was expressed in CTA per A_{280} reading instead of per unit weight of protein. The crude UK powder was dissolved in 0.05 *M* sodium acetate buffer containing 0.01% Tween 80 (pH 4.5). A liter of solution was prepared at the concentration of 3 mg/ml. The solution was applied to the SP cartridge directly without further filtration in order to eliminate the possible loss of enzymic activity in later steps. The addition of 0.01% Tween 80 to the buffer solutions improved the recovery. The selection of pH 4.5 as the optimum for UK adsorption on the SP matrix was based on the high isoelectric point of UK (ca. 9) [13] and the low pK_a value of the SP matrix (ca. 2.5) from the potentiometric titration.

Results shown in Fig. 3 indicate that 98% of the UK activity was bound on

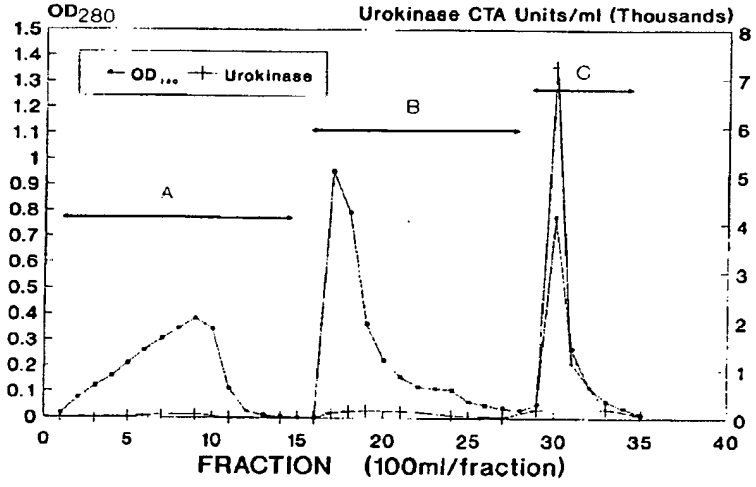

Fig. 3. Urokinase purification by SP cation exchange chromatographic cartridge. Crude UK (3.0 mg/ml, specific activity 1130 CTA U per A_{280}) was dissolved in 1 l of 0.05 *M* sodium acetate (pH 4.5) containing 0.01% Tween 80. The enzyme solution was applied to a 100-ml nominal-size SP cartridge equilibrated with the same buffer at 100 ml/min. The enzyme was first eluted by adding 0.4 *M* sodium chloride to the same buffer (specific activity 363 CTA U per A_{280}) as shown in region B. A 6.5-fold increase in specific activity of 7330 CTA U per A_{280} was eluted from the cartridge by adding 1.0 *M* sodium chloride to the buffer. (A) Unbound starting material washed to baseline with 0.05 *M* sodium acetate (pH 4.5) containing 0.01% Tween 80. (B) Pre-elution with 0.05 *M* sodium acetate (pH 4.5) containing 0.01% Tween 80 and 0.4 *M* sodium chloride. (C) Elution with 0.05 *M* sodium acetate (pH 4.5) containing 0.01% Tween 80 and 1.0 *M* sodium chloride.

the matrix. The 0.4 *M* calcium chloride pre-elution removed 70% of the bound proteins, of which 9% were enzymically active. The second step elution by 1.0 *M* sodium chloride added to the buffer recovered 80% of the bound enzymic activity and achieved a 6.5-fold increase in specific enzyme activity in this single step. At pH values higher than 4.5, the adsorption of UK by SP functional groups was found to be more effective but less selective. A balance between efficiency and yield leads to a pH of ca. 4.5. For further improvement in UK purity, a fast-flow zinc-chelated affinity cartridge was developed and applied in series with the SP cartridge. By applying the eluent collected from the SP cartridge at fractions number 30 and 31, containing $53.5 \cdot 10^4$ CTA U, through a 100-ml cartridge, we obtained a further 3.3-fold increase in specific activity with a yield of 89% (Fig. 4). The overall results (Table I) show a greater than 18-fold improvement in specific activity of UK with an 80% recovery of enzymic activity by passing the crude UK successively through an SP cation exchanger and a zinc-chelated affinity cartridge. It is well known that metal ions can form complexes with electron-rich compounds, such as aromatic or pseudoaromatic molecules, and may coordinate with O, N, and S atoms of amino acids [30].

The technical problem in metal immobilization is to attack the metal ions to a solid surface sufficiently tightly that they remain in place during the equi-

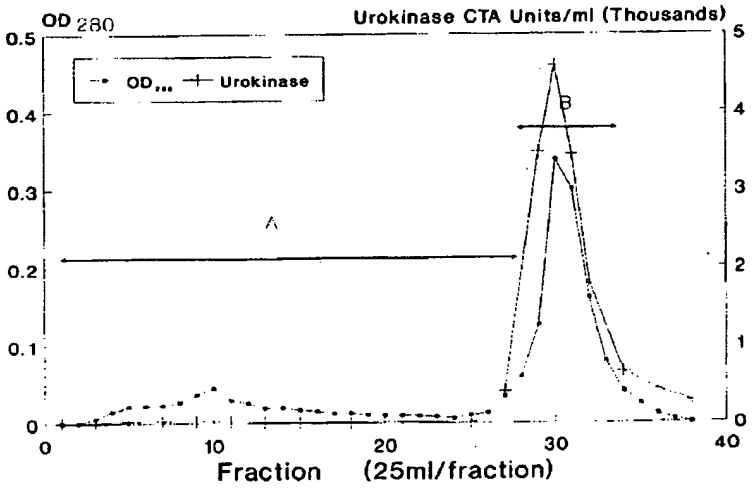

Fig. 4. Urokinase purification by zinc-chelated affinity chromatographic cartridge. UK pre-purified on an SP cartridge and collected from fractions 30 and 31 as shown in Fig. 3 was dialyzed and dissolved in 0.05 *M* borate buffer (pH 8.2) containing 0.2 *M* sodium chloride and 0.01% Tween 80. The enzyme solution was applied to a 100-ml nominal-size zinc-chelated cartridge pre-equilibrated with same buffer at 50 ml/min. The enzyme eluted by adding 0.05 *M* imidazole to the same buffer showed specific activity of 20 652 CTA U per A_{280}. (A) Unbound SP pre-purified urokinase washed to baseline with 0.05 *M* borate buffer (pH 8.2) containing 0.2 *M* sodium chloride and 0.01% Tween 80. (B) Elution with 0.05 *M* borate buffer (pH 8.2) containing 0.2 *M* sodium chloride, 0.01% Tween 80 and 0.05 *M* imidazole.

TABLE I

SUMMARY OF THE PURIFICATION OF CRUDE UROKINASE FROM URINE

Purification step	Volume (ml)	Total protein (A_{280} U)	Total urokinase (CTA U)	Specific activity (CTA U/A_{280})	Purification (x-fold)	Yield (%)
Crude urokinase powder dissolved in buffer	1000	1078	$121.9 \cdot 10^4$	1130	1.0	100
Cation-exchange SP cartridge						
(a) Collected both elution 1 and 2	1300	436	$106.3 \cdot 10^4$	2438	2.2	89.3
(b) Elution 2 only	300	130	$95.2 \cdot 10^4$	7323	6.5	78.1
unbound UK in filtrant		239	$2.5 \cdot 10^4$	105		(If discount the filtrant and elution 1)
In chelated affinity cartridge	170 of fraction 30 and 31 from elution 2	73.1	$53.5 \cdot 10^4$	7323	6.5	89.3
Elution with 0.05 *M* imidazole buffer	150	23	$47.5 \cdot 10^4$	20 652	18.3	$89.3 \times 88.8 - 79.3$

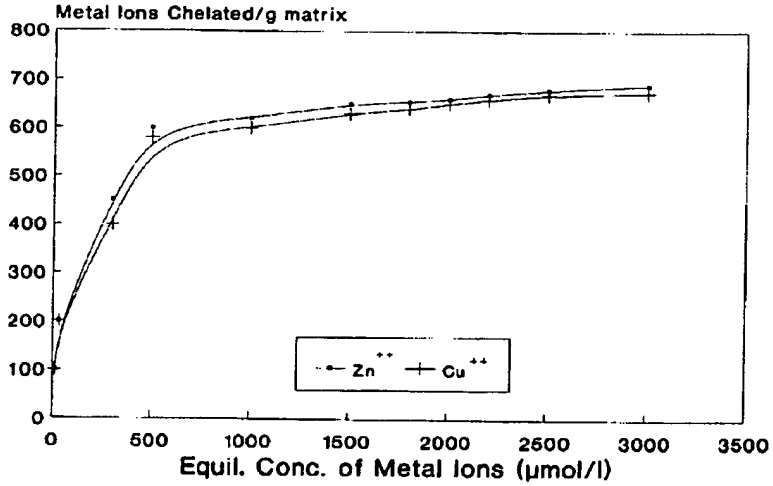

Fig. 5. Equilibrium concentration of metal ions (μmol/l). A 100-ml volume per tube of zinc nitrate hexahydrate or copper nitrate hemipentahydrate of different molarity were prepared by dissolving in deionized water at pH 5.0; 0.25 g of matrix was dispersed in each solution at room temperature with overnight shaking to ensure equilibration of the chelation metal ions.

libration and binding steps, yet can be reversibly stripped from the matrix by elution buffers during later stages. The chelation functional groups on the solid matrix must allow the metal ions to interact with the protein molecules to be purified. Therefore, the length of the spacer arm dictates the flexibility of the

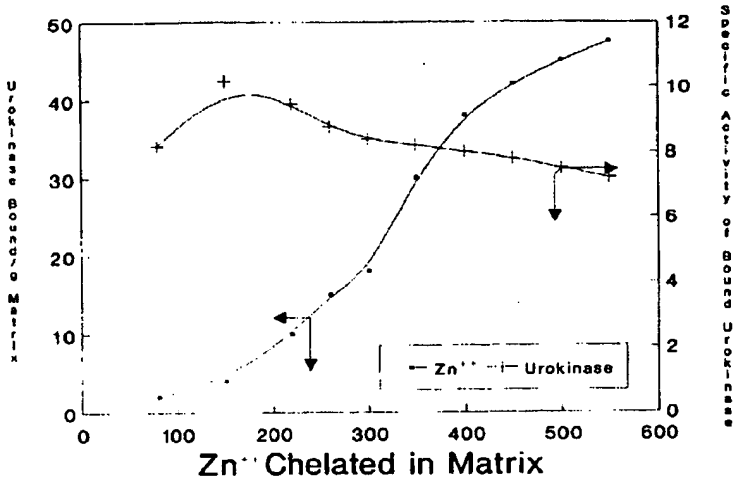

Fig. 6. Effect of chelated zinc concentration in the matrix on UK binding. A 10-ml sample of 4 mg/ml crude UK dissolved in 0.05 *M* borate buffer combined with 0.2 *M* sodium chloride and 0.01% Tween 80 (pH 8.2) was mixed with 50 mg of zinc-chelated matrix for 30 min at room temperature.

chelation functional groups, and the matrix porosity governs the accessibility of these groups to metal ions and the penetration of the biomolecules for the interaction to take place in a later stage. The conditions that affect the strength of interaction also play an important part in the process. The spacer arm of the dicarboxyl groups in the matrix of the present study is eight atoms in the acrylic polymer structure described previously. More than 650 μmol of zinc atoms determined by atomic adsorption can be chelated per gram of media, as shown in Fig. 5.

The relationship between the mount of zinc chelated to the matrix and the binding capacity of UK is shown in Fig. 6. It is plausible to interpret the gradual plateau in UK binding at zinc concentrations over 500 μmol/g of matrix as due to porosity limitations. The relatively large UK molecules may not be able to interact with the zinc atoms located in the concealed region of the matrix. The highly concentrated zinc ligands show another defect of reduced selectivity in the plot of specific activity of UK. Proteins other than UK may also bind to the chelated zinc at the higher concentrations, leading to the decrease in specific activity of UK when it is eluted from the matrix. Since the matrix is in paper form and has a density close to that of ordinary paper, it is dimensionally stable, especially chelating the carboxyl groups with zinc. The added advantage of radial-flow design permits the zinc-chelated cartridge to operate under high liquid flux with low-pressure build-up. The reported flow-rates of 50 and 100 ml/min on a 100-ml cartridge can be further increased by using a larger cartridge [28]. The application of a salt-eluted protein solution from the ion-exchange cartridge directly to the metal-chelated cartridge further simplifies the system. The effective elution of UK by 0.05 *M* imidazole buffer without further adjustment of pH or salt concentration demonstrates the advantage of

metal-chelating affinity chromatography as a means of purifying UK in a large-scale operation outside the laboratory.

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